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Zoonotic bacterial populations, gut fermentation characteristics and methane production in feedlot steers during oral nitroethane treatment and after the feeding of an experimental chlorate product ☆

Hector Gutierrez-Bañuelos[a], Robin C. Anderson[b,*], Gordon E. Carstens[a], Lisa J. Slay[a], Nicole Ramlachan[b], Shane M. Horrocks[b], Todd R. Callaway[b], Thomas S. Edrington[b], David J. Nisbet[b]

[a]*Department of Animal Science, Texas A&M University, College Station, TX 77843, USA*
[b]*United States Department of Agriculture, Agricultural Research Service, Southern Plains Agricultural Research Center, Food & Feed Safety Research Unit, College Station, TX 77845, USA*

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Abstract

Nitroethane inhibits the growth of certain zoonotic pathogens such as *Campylobacter* and *Salmonella* spp., foodborne pathogens estimated to cause millions of human infections each year, and enhances the *Salmonella*- and *Escherichia coli*-killing effect of an experimental chlorate product being developed as a feed additive to kill these bacteria immediately pre-harvest. Limited studies have shown that nitroethane inhibits ruminal methane production, which represents a loss of 2–12% of the host's gross energy intake and contributes to global warming and destruction of the ozone layer. The present study was conducted to assess the effects of 14-day oral nitroethane administration, 0 (0X), 80 (1X) or 160 (2X) mg nitroethane/kg body weight per day on ruminal and fecal *E. coli* and *Campylobacter,* ruminal and fecal methane-producing and nitroethane-reducing activity, whole animal methane emissions, and ruminal and fecal fermentation balance in Holstein steers ($n = 6$ per treatment) averaging 403 ± 26 (SD) kg BW. An experimental chlorate product was fed the day following the last nitroethane administration to determine effects on *E. coli* and *Campylobacter*. The experimental chlorate product decreased ($P < 0.001$) fecal, but not ruminal ($P > 0.05$) *E. coli* concentrations by 1000- and 10-fold by 24 and 48 h, respectively, after chlorate feeding when compared to pre-treatment concentrations ($> 5.7 \log_{10}$ colony forming units/g). No effects ($P > 0.05$) of nitroethane or the experimental chlorate product were observed on fecal *Campylobacter* concentrations; *Campylobacter* were not recovered from ruminal contents. Nitroethane treatment decreased ($P < 0.01$) ruminal (8.46, 7.91 and 4.74 ± 0.78 μmol/g/h) and fecal (3.90, 1.36 and 1.38 ± 0.50 μmol/g/h) methane-producing activity for treatments 0X, 1X and 2X, respectively. Administration of nitroethane increased ($P < 0.001$) nitroethane-reducing activity in ruminal, but not fecal samples. Day of study affected ruminal ($P < 0.0001$) but not fecal ($P > 0.05$) methane-producing and nitroethane-reducing activities ($P < 0.01$); treatment by day interactions were not observed ($P > 0.05$). Ruminal accumulations of acetate decreased ($P < 0.05$) in 2X-treated steers when compared with 0X- and 1X-treated steers, but no effect ($P > 0.05$) of nitroethane was observed on propionate, butyrate or the acetate to propionate ratio. Whole animal methane emissions, expressed as L/day or as a proportion of gross energy intake (%GEI), were unaffected by nitroethane treatment ($P > 0.05$), and were not correlated ($P > 0.05$) with ruminal methane-producing activity. These results demonstrate that oral nitroethane administration reduces ruminal methane-producing activity but suggest that a microbial adaptation, likely due to an *in situ* enrichment of ruminal nitroethane-reducing bacteria, may cause depletion of nitroethane, at least at the 1X administration dose, to concentrations too low to be

Abbreviations: BW, body weight; DMI, dry matter intake; NEg, net energy for gain; NEm, net energy for maintenance; TDN, total digestible nutrients.

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*Corresponding author. Tel.: +1 979 260 9317; fax: +1 979 260 9332.
E-mail address: anderson@ffsru.tamu.edu (R.C. Anderson).

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effective. Further research is warranted to determine if the optimization of dosage of nitroethane or related nitrocompouds can maintain the enteropathogen control and anti-methanogen effect in fed steers.
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1. Introduction

The bovine gastrointestinal tract is a recognized reservoir for enterohemorrhagic *Escherichia coli*, *Salmonella* and, to a lesser extent, *Campylobacter*; bacterial pathogens estimated to cause more than 3.9 million human infections annually [1]. Human infections by these bacteria are estimated to cost more than $4.5 billion each year [2]. Quantitative risk assessments indicate that strategies that can reduce concentrations of these bacteria in cattle before they arrive at slaughter plants may significantly reduce human exposures to the pathogens [3,4]. Several such strategies are currently being investigated, including vaccination [5], the administration of competitive exclusion or colicin-producing *E. coli* [6–8], probiotic *Lactobacillus* spp. [9,10], neomycin [11] and an experimental chlorate product [12–15]. Additionally, nitroethane has shown inhibitory activity against *Salmonella* and *Listeria* in ruminal contents *in vitro* [16] as well as against *Salmonella* and *Campylobacter* in swine [17,18]. Moreover, nitroethane, or related nitrocompounds, have been shown to significantly enhance the *Salmonella*- and *E. coli*-killing activity of chlorate in swine gut contents *in vitro* and *in vivo* [17,19].

While initial results with these aforementioned interventions have been promising, there remains a need to make such strategies economically acceptable for cattle feeders because they likely will be expected to absorb the costs of implementing these interventions. In that regard, the recent work with the nitrocompounds may have applications as these compounds are potent inhibitors of ruminal methanogenesis. Methanogenesis, with its concomitant consumption of hydrogen, plays an important role in maintaining a low partial pressure of hydrogen within the ruminal microbial ecosystem which allows fermentation to proceed largely unencumbered by the accumulation of excess reducing equivalents [20]. Despite this beneficial role, methane production is recognized as an energetically wasteful process to ruminants, resulting in the loss of 2–12% of the gross energy consumed by the animal [21]. Methane is also a greenhouse gas that has been implicated in contributing to global warming and ozone layer destruction [22]. Numerous strategies for reducing energetic losses associated with ruminal methane production have been investigated but the majority of these techniques or products not only inhibited methane production, but also inhibited the beneficial oxidation of hydrogen affected by this process [22]. Changes produced by these inhibitors include reduction in certain digestive process, microbial growth yields, a decreased production of acetate and an increased production of reduced fermentation acids, notably propionate [23]. Moreover, inhibition of methane production by these inhibitors often appears to be transient due to the ability of the rumen ecosystem to adapt to ecological perturbations [24]. In contrast, results so far from two studies have shown that the methane-inhibitor, nitroethane, had little effect on amounts or molar proportions of volatile fatty acids (VFA) produced within *in vitro* incubations or the ovine rumen thus indicating that this inhibitor may conserve fermentative efficiencies associated with microbial interspecies hydrogen transfer reactions [25,26].

The objectives of this study were to evaluate the effect of oral nitroethane administration on select fermentation variables and zoonotic bacterial populations in growing steers over a 14-day treatment period both prior to and following a single day feeding (day 15) of an experimental chlorate product.

2. Materials and methods

2.1. Experimental design

Eighteen Holstein steers averaging 403 ± 26 (mean $\pm$ SD) kg body weight (BW) were acclimated over a 21-day period a diet containing 50% dry rolled corn, 25% chopped alfalfa, 13% cotton seed hulls, 7% molasses, 3% soybean meal (49% crude protein), and 2% premix (30.26% dry rolled corn, 0.5% COOP Beef TM, 2.5% ADE, 4.56% Vitamin E, 27.33% urea, 14.85% Limestone, and 20% salt). The NRC predicted nutrient profile (@ 3.0% BW dry matter intake, DMI) was: dry matter, 89%; TDN, 71%; NEm, 1.65 Mcal/kg; NEg, 1.03 Mcal/kg; crude protein, 13.3%; calcium, 0.51% and phosphorus, 0.24%. Steers were randomly allocated ($n = 6$/treatment) to one of the following treatments: 0, 80 or 160 mg nitroethane/kg BW per day (corresponding to 0X, 1X and 2X treatments, respectively). Steers were penned separately and provided ad libitum access to the study diet which was fed in two equal sized meals at 08:00 and 16:30. Feed not consumed was recovered and intake was calculated as the difference between dry matter offered and refused. Because *Campylobacter* and *Salmonella* prevalence in feedlot steers can be quite variable, all steers were orally inoculated 4 days prior to initiation of treatments with 20 mL of a pooled suspension of freshly collected feces (prepared by combining 10 g feces obtained from each steer with 1 L of phosphate buffer, pH 7.0). Bacteriological cultivation of portions of rumen or fecal samples collected 4 days prior to initiation of treatment revealed that 9 steers

were colonized by *Campylobacter* and only one steer was colonized by *Salmonella*. Consequently, in order to provide a better *Salmonella*-challenge, the isolated *Salmonella* was grown overnight at 37 °C grown in Tryptic Soy broth (Becton Dickinson Microbiological Systems, Sparks, MD, USA) and orally inoculated to each steer (9×10^9 CFU/steer) immediately following collection of rumen and fecal samples the day immediately prior to initiation of treatments (day −1).

Upon initiation of treatments, nitroethane was administered as the sodium salt [27] twice daily (08:00 and 16:00) by oral gavage. Gavage volumes ranged from 146.2 to 353.1 mL per day depending on dose and individual steer body weight. Control steers were administered buffer alone at the same volume basis as steers administered the 2X nitroethane treatment. Ruminal fluid collected by stomach tube and freshly voided feces were collected approximately 2 h after the morning feeding on days −1, 1, 2, 7 and 14 relative to nitroethane treatment. Specimens were placed immediately into serum vials (ruminal fluid) or whirlpac bags (feces) which were then closed and returned to the laboratory within 1–2 h for determinations of VFA concentrations, methane-producing and nitroethane-reducing activities and for bacteriological cultivation. Whole animal methane emissions were measured in exhaled gases collected from 09:00 to 07:00 using the sulfur hexafluoride tracer gas technique [28]. Pre-evacuated collection canisters were placed on the steers before the morning feeding and were removed after 22 h.

One day after the end of the 14 day nitroethane treatment period, all steers were fed a proprietary experimental chlorate product at 140 mg/kg BW (EKA Chemicals Inc., Marietta, GA, USA) in their last meal and ruminal fluid and feces were again sampled 24 (day 16) and 48 h (day 17) later to determine effects on ruminal and fecal bacteria.

2.2. Analytical procedures

The gas samples were analyzed by gas chromatography to measure methane and sulfur hexafluoride concentrations [28]. VFA concentrations were measured by gas chromatography [29] and estimates of methane produced were derived from the fermentation balance of Wolin [30]. Methane-producing activity was determined by *in vitro* incubation of 5 mL ruminal fluid or 2 g feces, mixed with 5 or 8 mL, respectively, anaerobic dilution solution [31] containing 60 mM sodium formate and 0.2 g finely ground alfalfa (to pass a 4 mm screen). The tubes were capped and incubated 3 h at 39 °C under a hydrogen:carbon dioxide (50:50 mix) atmosphere. At the end of the incubation period, methane concentration was determined by gas chromatography [32]. Nitroethane-reducing activity was determined in separate incubations conducted similarly except containing 10 mM added nitroethane; fluid samples collected at 0, 3, 6 and 24 h were analyzed for nitroethane colorimetrically [27]. Quantitative cultivation of indigenous *E. coli*, coliforms, *Campylobacter* spp. and *Salmonella* spp. was achieved via plating of serial 10-fold dilutions (in phosphate buffer pH 6.5) to 3 M *E. coli*/Coliform Count petrifilm (3 M Microbiology Products, St. Paul, MN, USA), Campy Cefex agar [33], or Brilliant Green agar (Oxoid LTD, Basingstoke, Hampshire, UK), respectively. Inoculated petrifilm and Brilliant green agar were incubated at 37 °C for 24 h. Inoculated Campy Cefex agar was incubated at 37 °C 48 h under an microaerophilic gas (10% CO_2, 5% O_2, and 85% N_2). Qualitative cultivation of *Salmonella* was accomplished via overnight enrichment in Tetrathionate broth (Becton Dickinson Microbiology Systems) and further enrichment for 18–24 h in Rappaport-Vassiladias R10 broth (Becton Dickinson Microbiology Systems) and selective differentiation on Brilliant Green Agar [34]. Recovered *Salmonella* were serotyped at the National Veterinary Services Laboratory (Ames, IA, USA). Samples were enriched and cultured for *E. coli* O157:H7 using immunomagnetic separation [35].

2.3. Statistical analysis

Methane-producing activity, nitroethane-reducing activity, whole animal methane measurements, VFA concentrations, ratio of acetate to propionate, estimated methane production, and $\log_{10}$ transformations of *E. coli* and *Campylobacter* colony forming units (CFU) were analyzed for effects nitroethane treatment, day of treatment, and their interaction using a repeated measures analysis of variance. Means were further separated using a least significant difference procedure. Due to the magnitude of inter-animal variation in whole animal methane emissions data, a covariate analysis of variance was conducted using day 0 methane measurements as the covariate for day 7 and 14 measurements. Daily DMI and average daily gain at the end of the 14-day nitroethane treatment period were analyzed by a completely randomized analysis of variance. Tests for the degree of linear association between measures of whole animal methane emissions, methane-producing activity and estimations of methane production via fermentation balance were accomplished by Pearson correlation.

3. Results

3.1. Ruminal and fecal enterobacteria

E. coli O157:H7 were not recovered from any of the ruminal fecal specimens collected on day −1 of the study, thus none of the subsequent samples were cultured for this bacterium. Moreover, *Salmonella* were recovered only from enriched specimens indicating that concentrations were below our limit of detection (<10 CFU/g of contents). *Salmonella* were recovered from ruminal and fecal specimens collected on day −1 from 12 of the 18 steers, but from feces of only 1 steer at the end of the 14 day of nitroethane treatment. Following the experimental

chlorate treatment, *Salmonella* were undetectable in ruminal or fecal specimens. For the *Salmonella* isolated pre-nitroethane treatment, 10 were identified as *Salmonella enterica* serovar Heidelberg, which was the serovar initially isolated and inoculated into all steers on the day immediately preceding initiation of nitroethane administration. The other two isolates were identified as *Salmonella enterica* serovars Anatum and Typhimurium. Two of these isolates (one Heidelberg isolate and the Typhimurium isolate) were recovered from steers allocated to receive the 0X treatment, five of these isolates (all identified as Heidelberg) were recovered from steers allocated to receive the 1X treatment, the remaining 5 isolates (4 identified as Heidelberg and the remaining as Anatum) were recovered from steers to receive the 2X treatment. A single *Salmonella* isolate, identified as serovar Heidelberg, was recovered from feces of 1 steer (from the 2X treatment group) at the end of nitroethane-treatment. Because of the small number of animals in this study and the pattern of qualitative *Salmonella* recovery, these results were not statistically analyzed but rather are presented descriptively only.

Nitroethane treatment had no effect on ruminal or fecal concentrations of generic *E. coli* or *Campylobacter* (Table 1). *Campylobacter* spp. were not detected in any of the ruminal fluid samples at any time and were highest ($P<0.05$) before initiation of nitroethane administration (Table 1). An effect of day of treatment was observed on fecal ($P<0.05$) but not ruminal ($P>0.05$) concentrations of generic *E. coli*, with concentrations being lower on days 16 and 17 of the study (corresponding to 1 and 2 days post-chlorate treatment given on day 15) than before (day −1) or at the end (day 14) of the nitroethane feeding period (Table 1). No interaction between chlorate and nitroethane was observed on fecal *E. coli* populations.

Table 1
Effect of oral nitroethane administration and chlorate feeding on ruminal and fecal bacterial concentrations in fed steers

Nitroethane treatment (mg nitroethane/kg body weight per day)	Generic *Escherichia coli* ($\log_{10}$ CFU/g contents)		*Campylobacter* ($\log_{10}$ CFU/g contents)	
	Ruminal	Fecal	Ruminal	Fecal
0 (0X)	3.33	4.59	0	2.92
80 (1X)	3.54	4.86	0	3.01
160 (2X)	3.57	4.78	0	1.43
P-value	0.7210	0.6536	—	0.2267
SEM	0.23	0.21	—	0.69
Time of treatment (*day*)				
−1 (1 d pre-nitroethane treatment)	3.26	5.76[a]	0	1.68[b]
14 (at end of nitroethane treatment)	3.41	5.92[a]	0	2.69[a]
16 (1 day post-chlorate treatment)	3.56	2.59[c]	0	2.65[a]
17 (2 day post-chlorate treatment)	3.70	4.71[b]	0	2.80[a]
P-value	0.1663	0.0001	—	0.0001
SEM	0.14	0.18	—	0.17
Interaction				
P-value	0.9518	0.9555	—	0.9887
SEM	0.25	0.31	—	0.30

[a,b,c]Means within columns with unlike superscripts differ ($P<0.05$).

3.2. Ruminal and fecal methane-producing and nitroethane-reducing activity, whole animal methane emissions and animal performance

Oral nitroethane administration decreased ($P<0.05$) ruminal methane-producing activity, with the activity in steers administered the 2X nitroethane treatment being 40% lower than that observed in steers administered the 0X or 1X nitroethane treatment (Table 2). An effect of day of treatment ($P<0.05$) was observed, with the lowest ruminal methane-producing activity occurring on day 2 and the highest activity occurring on days 7 and 14 of nitroethane administration (Table 2). Main effects of nitroethane treatment, day of treatment (Table 2) and their interaction on ruminal nitroethane-reducing activity were observed (Fig. 1).

Fecal methane-producing activity was also decreased ($P<0.05$) due to nitroethane administration, with main effect means for steers administered the 1X and 2X nitroethane treatment being more than 60% lower than that observed in steers administered 0X nitroethane (Table 2). This later finding suggests the passage of effective concentrations of nitroethane to the lower gut. No treatment by day of treatment interaction was observed

Table 2
Effect of oral nitroethane administration on methane-producing and nitroethane-reducing activity in fed steers

Treatment (g nitroethane/kg body weight per day)	Methane-producing activity (μmol methane/g contents per h)		Nitroethane-reducing activity (μmol nitroethane/g contents per h)	
	Ruminal	Fecal	Ruminal	Fecal
0 (0X)	8.46[a]	3.90[a]	0.05[b]	0.05
80 (1X)	7.91[a]	1.36[b]	0.15[a]	0.07
160 (2X)	4.74[b]	1.38[b]	0.13[a]	0.07
P-value	0.0084	0.0033	0.0005	0.4714
SEM	0.78	0.50	0.02	0.01
Time of treatment (*day*)				
−1 (1 day pre-nitroethane treatment)	7.44[a,b]	3.11	0.04[b]	0.06
1	6.26[a,b]	1.66	0.12[a]	0.06
2	5.16[c]	2.25	0.11[a]	0.06
7	8.50[b,c]	2.44	0.14[a]	0.08
14 (at end of nitroethane treatment)	7.82[b,c]	1.61	0.15[a]	0.08
P-value	0.0028	0.1184	< 0.0001	0.2698
SEM	0.62	0.45	0.02	0.01
Interaction				
P-value	0.1797	0.9376	0.0003	0.8046
SEM	1.08	0.77	0.03	0.02

[a,b,c]Values within columns with unlike superscripts differ ($P<0.05$).

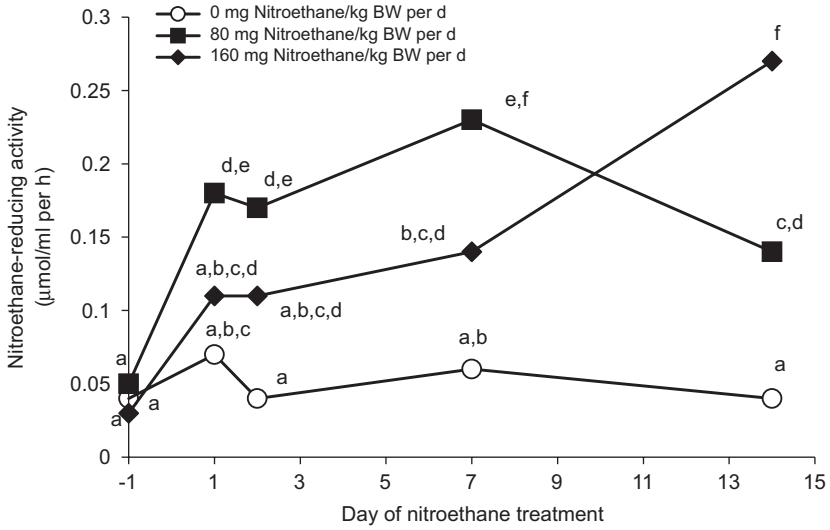

Fig. 1. Effects of oral nitroethane administration on ruminal nitroethane-reducing activity in fed steers. Nitroethane was administered twice daily (08:00 and 16:00) via oral gavage. A repeated measures analysis of variance revealed a treatment by day of treatment interaction ($P = 0.0003$; SEM = 0.03), means with unlike superscripts differ at $P < 0.05$.

Table 3
Effect of oral nitroethane administration on ruminal fermentation balance in fed steers

Treatment (g nitroethane/kg body weight per day)	Ruminal concentrations (μmol/g contents)				Ratio of acetate to propionate
	Acetate	Propionate	Butyrate	Estimated methane	
0 (0X)	45.53[a]	18.83	10.44	23.3	2.55
80 (1X)	42.96[a]	18.76	12.25	22.9	2.39
160 (2X)	34.61[b]	15.60	11.38	19.1	2.17
P-value	0.0169	0.1515	0.3257	0.0627	0.1491
SEM	2.54	1.00	0.74	1.34	0.12
Time of treatment (*day*)					
−1 (1 day pre-nitroethane treatment)	24.42[c]	15.77[b]	9.20[b]	12.8[b]	1.68[c]
1	35.91[b]	17.15[a,b]	9.91[b]	18.6[b]	2.14[b,c]
2	37.51[b]	15.62[b]	10.36[b]	20.0[b]	2.51[a,b]
7	55.66[a]	19.70[a]	14.52[a]	30.2[a]	2.81[a]
14 (at end of nitroethane treatment)	51.67[a]	20.41[a]	12.82[a]	27.1[a]	2.7[a]
P-value	< 0.0001	0.0200	0.0002	< 0.0001	0.0005
SEM	2.98	1.28	0.88	1.70	0.19
Interaction					
P-value	0.2374	0.2280	0.6166	0.3446	0.5503
SEM	5.50	2.36	1.63	3.14	0.35

[a,b,c]Values within columns with unlike superscripts differ ($P < 0.05$).

on either ruminal or fecal methane-producing activity. No effects of nitroethane treatment, day of treatment or their interaction ($P > 0.05$) were observed on fecal nitroethane-reducing activity (Table 2).

Whole animal methane emissions (±SE), whether expressed as liters produced/day (290.5, 239.1 vs. 112.0 ± 38.2 L/day for 0X, 1X and 2X treatments, respectively), or as methane energy as a percentage of gross energy intake (4.31, 3.63 and 4.05 ± 0.44% GEI for 0X, 1X and 2X treatments, respectively), were not affected ($P > 0.05$) by nitroethane treatment. Daily DMI and average daily gain were not affected ($P > 0.05$) by treatment and averaged (±SE) 15.0 ± 0.5 and 1.28 ± 0.20 kg/day, respectively, over the 14 day nitroethane treatment period. An effect of day of treatment ($P < 0.05$), but not a treatment by day interaction was observed for whole animal methane emissions. Methane emissions were lower on day 14 (3.8 ± 0.23% GEI) of the study compared to day 7 (4.3 ± 0.23% GEI).

3.3. Ruminal and fecal fermentation balance

Acetate concentrations were lower ($P < 0.05$) in ruminal fluid collected from steers administered the 2X nitroethane treatment compared to steers administered the 0X or 1X nitroethane treatments (Table 3). Propionate and butyrate

Table 4
Effect of oral nitroethane administration on fecal fermentation balance in fed steers

Treatment (g nitroethane/kg body weight per day)	Fecal concentrations (μmol/g contents)				Ratio of acetate to propionate
	Acetate	Propionate	Butyrate	Estimated methane	
0 (0X)	59.08	25.72	20.09	33.2	2.39
80 (1X)	67.69	29.17	20.45	36.8	2.51
160 (2X)	58.24	25.82	19.89	32.6	2.33
P-value	0.2739	0.5658	0.9799	0.4493	0.6212
SEM	4.36	2.37	2.03	2.44	0.08
Time of treatment (*day*)					
−1 (1 day pre-nitroethane treatment)	76.51[a]	30.62[a,b]	20.54	40.9[a]	2.64[a]
1	64.11[a,b]	25.11[b,c]	20.07	35.8[a,b]	2.56[a]
2	52.05[b]	21.26[c]	16.97	29.2[b]	2.50[a]
7	54.67[b]	25.09[b,c]	19.94	31.0[b]	2.33[a,b]
14 (at end of nitroethane treatment)	61.02[b]	32.43[a]	23.20	34.0[a,b]	2.01[b]
P-value	0.0079	0.0189	0.1296	0.0271	0.0070
SEM	4.92	2.59	1.67	2.65	0.13
Interaction					
P-value	0.9380	0.7845	0.9712	0.9924	0.4146
SEM	9.08	4.78	3.08	4.89	0.24

[a,b,c]Values within columns with unlike superscripts differ ($P<0.05$).

concentrations, as well as the ratio of acetate to propionate were unaffected by nitroethane treatment (Table 3). Estimates of methane production derived from a fermentation balance tended to be lowest ($P<0.07$) in ruminal fluid from steers administered the 2X nitroethane treatment than in fluid collected from steers administered the 0X and 1X nitroethane treatments (Table 3). Fecal VFA accumulations or estimated fecal methane production were not affected ($P>0.05$) by nitroethane treatment (Table 3).

An effect of day of nitroethane treatment was observed ($P<0.05$) on ruminal VFA concentrations, with concentrations as well as the acetate to propionate ratio and estimated methane production generally being higher on days 7 and 14 of nitroethane treatment than earlier days of the study (Table 3). In contrast, fecal acetate concentrations were lowest ($P<0.05$) on days 2, 7 and 14 of nitroethane treatment and fecal propionate was lowest ($P<0.05$) on day 2 and highest ($P<0.05$) on day 14 of treatment (Table 4). Fecal butyrate concentrations were unaffected ($P>0.05$) by day of treatment but the ratio of acetate to propionate was lowest ($P<0.05$) on day 14 of treatment and the amount of estimated methane produced was lowest on days 2 and 7 of treatment (Table 4). Treatment by day of treatment interactions were not observed ($P>0.05$) for ruminal or fecal VFA accumulations, ratios of acetate to propionate or estimated methane production.

4. Discussion

In agreement with earlier reports [12–15], results from the present study demonstrate that feeding an experimental chlorate product reduced ($P<0.05$) generic *E. coli* concentrations in feces >1000-fold by 24 h post-chlorate treatment (Table 1). Moreover, these results provide additional information pertaining to the persistence of the bactericidal effect of chlorate by revealing that *E. coli* concentrations were reduced in the lower gut by 48 h post-chlorate treatment, albeit only 10-fold lower than pre-treatment concentrations (Table 3). This diminishing effect of chlorate over time is not unexpected; however, as chlorate exerts its effects by being catalytically reduced, and thus depleted, by membrane bound respiratory nitrate reductase (Nar), possessed by bacteria such as *E. coli* and *Salmonella* [36]. No effect of the experimental chlorate treatment ($P>0.05$) was observed on ruminal *E. coli* concentrations although this was expected as the chlorate product had been reported to possess rumen bypass characteristics [37].

Unlike that observed with swine, where an additive *E. coli*- and *Salmonella*-killing effect was observed with combined or prior addition of nitroethane with chlorate [17,19], no interaction between chlorate and nitroethane was observed on generic *E. coli* populations in this study. Furthermore, unlike that observed earlier [18,38], nitroethane treatment in the present study had no effect against *E. coli* or *Campylobacter*. In those earlier studies, inhibition of *E. coli*, *Salmonella* or *Campylobacter* in swine gut contents was consistently observed at concentrations >10 mM. Consequently, we suspect that the nitroethane dose administered in this study was too low (approximately 8.6 mM ruminal nitroethane concentration based on the 2X dose and a 100 L rumen volume) to exert inhibitory activity against these bacteria. Alternatively, it is probable that some of the nitroethane may have been absorbed across the rumen wall, expired in eructated gases or consumed by gut bacteria such as the highly competent nitro-respiring *Denitrobacterium detoxificans* [39] thus

further depleting gut concentrations of this inhibitor. In support of the later hypothesis, ruminal nitroethane-reducing activity increased markedly soon after initiation of nitroethane administration (Fig. 1) and this likely contributed to depletion of nitroethane. No conclusions can be made regarding effects of nitroethane or experimental chlorate treatment on *E. coli* O157:H7, which were not detected pre-treatment, or on the incidence of *Salmonella,* which was reduced 80–100% from pre-treatment measurments regardless of treatments.

In agreement with results of a previous study [26], oral nitroethane administration reduced ($P<0.05$) ruminal methane-producing activity in this study (Table 2) and tended ($P<0.10$) to reduce the theoretical production of ruminal methane as estimated by fermentation balance. Ruminal methane-producing activity, which is an indirect measure of numbers of methanogens, and theoretical ruminal methane production were shown to be correlated (Pearson correlation coefficient = 0.3513, $P = 0.001$). In contrast to earlier results [40], however, whole animal methane emissions were not affected ($P>0.05$) by nitroethane treatment in this study, possibly due to the high variability in methane recovery and dry matter intake that was observed. As reported by others [41], high day to day variation within and between animals and problems with missing measurements were encountered in this study, problems that when compounded by the low numbers of experimental units may have limited the ability of the method to detect potential treatment effects. Moreover, pre-treatment emission measurements were lower for control steers than for 1X or 2X treated steers (Fig. 2) which further confounded the results. Additionally, no correlation was found between sulfur hexafluoride-derived whole animal methane emissions (%GEI) and ruminal methane-reducing activity (Pearson correlation coefficient = −0.1897, $P = 0.1965$) or theoretical production of ruminal methane (Pearson correlation coefficient = −0.0562, $P = 0.7046$). Wright and others

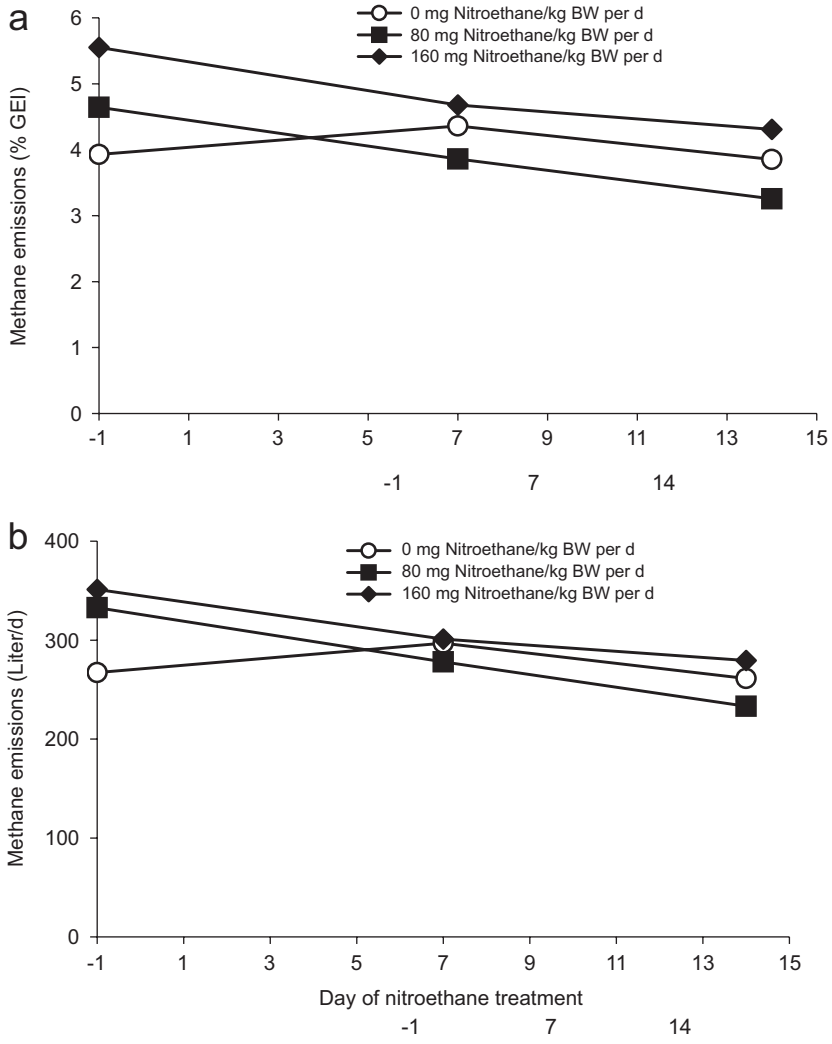

Fig. 2. Effects of oral nitroethane administration on ruminal methane emissions, when expressed as percent of gross energy intake (a) or liters/day (b), in fed steers. Nitroethane was adminstered twice daily (08:00 and 16:00) via oral gavage. A repeated measures analysis of variance revealed no treatment by day of treatment interaction ($P = 0.3447$ and 0.4157 for A and B, respectively; SEM = 0.41 and 29.34, respectively).

[41] found no correlation between indirect chamber calorimetry measurements of whole animal methane emissions and those obtained using the sulfur hexafluoride method. An effect of day of treatment ($P<0.05$) was observed on whole animal methane emissions, with the lowest production occurring on day 14 compared with that produced pre-treatment or on day 7. A day affect was also observed on methane-producing activity, but in this case reductions in activity diminished over time, suggesting the occurrence of an apparent adaptation within the rumen ecosystem. Ruminal adaptation to methane inhibitors is well known [22]. In the present study, the adaptation was mainly due to a transient decrease in methane-producing activity in ruminal contents from steers administered the 1X nitroethane treatment as the methane-producing activity in contents from steers administered the 2X treatment remained at least 30% lower than the activity measured pre-treatment (Fig. 3). An apparent adaptation was observed in the earlier study as well as in sheep administered 24 or 72 mg nitroethane/kg BW per day [26]. Adaptation likely occurs, at least in part, because of an enrichment of ruminal nitroethane-consuming bacteria as evidenced in the present study by main effects of nitroethane treatment, day of treatment (Table 2) and their interaction on ruminal nitroethane-reducing activity (Fig. 1). At present, *D. detoxificans*, an obligate non-fermentative nitro-respiring anaerobe, is the only ruminal bacterium known to possess appreciable ability to metabolize nitroethane, as well as a variety of other oxidized nitrocompounds, coupling their reduction to the oxidation of hydrogen, formate or lactate [39]. It is known that concentrations of this bacterium can be increased >1000-fold and rates of nitrocompound metabolism can be increased during growth with additions of a related nitrocompound, 3-nitro-1-propanol, the poisonous compound found in various species of the leguminous forage *Astragalus* (milkvetchs) [42]. Supplementing cattle diets with nitroethane also increased disappearance rates of ruminal 3-nitro-1-propanol [27,43]. In the present study, ruminal nitroethane-reducing activity increased to $>0.17\pm0.05$ μmol nitroethane/mL/h for steers administered the 1X nitroethane treatment indicating that more than 90% of their daily dose (estimated to be 4.3 μmol nitroethane/mL ruminal fluid per day) would have been consumed by 24 h. Thus, it is reasonable to expect that while effective methane-inhibiting concentrations of nitroethane may have been maintained in the steers administered the 2X nitroethane treatment, concentrations were probably depleted in the steers administered the 1X treatment. Results from *in vitro* incubations of ruminal contents have shown that the methane-inhibiting effect of nitroethane was reduced approximately 36% when nitroethane concentration was reduced from 12 to 2 mM [25].

Many methane inhibitors that directly inhibit methanogenic bacteria dissipate the hydrogen consuming role played by methanogens. This subsequently results in decreased accumulations of acetate and, as a compensatory route for dispensing of reducing equivalents, increased accumulations of more reduced fatty acids such as propionate and butyrate [22]. While the mechanistic effects of nitroethane on methanogens has yet to be determined, a direct chemical inhibition is likely, at least initially, as related compounds, 3-nitro-1-propionic acid and 3-nitro-1-propanol, were shown to inhibit *Methanobrevibacter ruminantium* and *Methanobrevibacter smithii* directly [44]. In this and an earlier study [26], however, administration of nitroethane, or a related compound, 2-nitro-1-propanol, at less than 80 mg/kg BW per day had no effect on ruminal accumulation of VFA. Acetate accumulation was decreased ($P<0.05$) in ruminal contents from sheep administered 120 mg 2-nitro-1-propanol [26] and in the steers in this study administered the 2X nitroethane treatment thus suggesting a potential detrimental effect of these higher doses. It is unlikely that nitroethane caused inhibitory

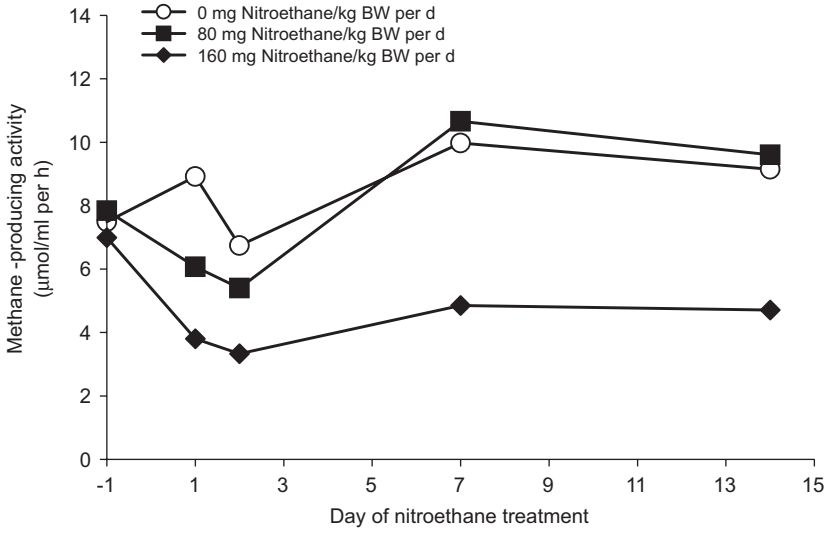

Fig. 3. Effects of oral nitroethane administration on ruminal methane-producing activity in fed steers. Nitroethane was administered twice daily (08:00 and 16:00) via oral gavage. A repeated measures analysis of variance revealed no treatment by day of treatment interaction ($P = 0.1797$; SEM = 1.08).

accumulations of hydrogen within the rumens of nitroethane-treated steers as reducing equivalents appeared not to be redirected to the production and accumulation of the more reduced acids, propionate and butyrate, which were unaffected ($P > 0.05$) by treatment (Table 3). This conclusion is further supported by the observation that the ratio of acetate to propionate was unaffected ($P > 0.05$) by nitroethane treatment (Table 3). Moreover, only small quantities of H_2 ($< 3\,\mu mol/mL$ ruminal fluid) were shown to accumulate *in vitro* incubations of ruminal fluid with nitroethane, 2-nitro-1-propanol or 2-nitroethanol [25]. The reduction of nitroethane would be expected to consume at least some of the reducing equivalents not used to reduce carbon dioxide to methane and as nitroethane-reducing activity increased more equivalents would be consumed. Thus, while nitrocompounds may initially exert a direct inhibition on methanogenic bacteria, inhibition of methane production could also progress to be partly competitive, as numbers of hydrogen-oxidizing bacteria like *D. detoxificans* increase, they may be able to outcompete methanogens for reducing equivalents. *In vitro* incubation of *D. detoxificans* strain NPOH1 in ruminal contents with added nitrate as an electron acceptor inhibited methane production by 94% [44]. Reducing equivalents may also be consumed to support anabolic cell processes and growth of increasing populations of nitroethane-reducing bacteria. In contrast to that observed with ruminal fluid, *in vitro* incubations of chicken cecal contents with nitroethane resulted in appreciable accumulations of hydrogen (more than $> 10\,\mu mol$ hydrogen/g cecal content) over that produced by of that of control cultures incubated without nitroethane [45]. It is not known whether or not chickens may be colonized with *D. detoxificans*, which if absent, could possibly explain why there was no apparent alternative hydrogen sink. Presently, *D. detoxificans* has only been isolated from ruminal contents [39,42] although *D. detoxificans*-like nucleic acid has been recovered from human dental caries [46].

As in an earlier study [26] ruminal fermentation efficiency appeared to increase during the study's progression as evidenced by an increase in ruminal VFA accumulations over time for all steers (Table 3) and this was coincident with decreases in fecal acetate and propionate (albeit transient) concentrations (Table 4). It is possible that this may have due to the buffering capacity of the alkaline (pH 10.2) phosphate buffer used as a placebo or carrier of nitroethane; however, this is unlikely as total daily amounts of buffer or treatment additions did not exceed 360 mL which, assuming a rumen volume of 100 L, is less than 0.4% total ruminal volume. The alkaline buffer may have affected increased ruminal digestion thereby decreasing substrate passage to the lower gut. If this is the case, then decreased substrate availability, rather than nitroethane per se, may be responsible for the decreased methane-producing activity observed in fecal contents of the nitroethane-treated steers in this study. Fecal nitroethane-reducing activity did not differ between control- and nitroethane-treated steers, but this was more likely due to reduced access to lower concentrations of nitroethane.

5. Conclusions

Results demonstrated that nitroethane administration reduced methane-producing activity, an indirect measure of numbers of methane-producing bacteria, in rumen contents of growing steers fed the mixed diet by more than 40% although these results were not corroborated by measurements of sulfur hexafluoride determination of whole animal methane emissions. Contrary to findings from numerous *in vitro* and *in vivo* studies in swine, our results did not support our hypothesis that nitroethane would reduce *Campylobacter* and *Salmonella* in these fed steers, possibly because rapid absorption, expiration and rumen degradation prevented accumulations of nitroethane to levels needed to be effective against these enteropathogens. Studies testing the related nitrocompounds, 2-nitroethanol and 2-nitro-1-propanol, which exert greater anti-*E. coli*, anti-*Salmonella* and anti-*Campylobacter* activity than nitroethane [19,38] as alternatives to nitroethane may be warranted, although in the case of 2-nitro-1-propanol, its methane-inhibiting activity was found to be inferior to that exerted by nitroethane [26]. Feeding chlorate in the last day's meal reduced ($P < 0.05$) fecal *E. coli* concentrations by up to 1000-fold but this was not enhanced by prior nitroethane treatment. Because an apparent microbial resistance could be operative, further work is needed to determine if dosage of nitroethane or related nitrocompounds can be optimized to achieve and maintain concomitant enteropathogen control and methane reduction in fed steers.

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